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DETECTION OF PORCINE DNA IN GELATIN CONTAINING MUFFINS USING PORCINE GENE CHIP™ ANALYSIS

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ABSTRACT

This study was carried out to compare the sensitivity of two detection techniques using conventional polymerase chain reaction (PCR) and Porcine gene chip (Olipro™, Selangor, MY). Experimental samples consist of 15 muffins added with different percentage of porcine gelatin (1, 0.5, 0.1, 0.05, 0.01 and 0% w/w), and then cooked in two different ways (steamed and baked) were analyzed for the presence of porcine DNA in gelatin. Both Porcine gene chip and PCR analysis were targeting the gene of cytochrome *b* (*cyt* b). Porcine DNA detection in muffin samples using gene chip and PCR method were able to detect the presence of the porcine DNA at 73 and 53% of samples, respectively. Thus, this study demonstrated that the sensitivity level of porcine the DNA detection was high in Porcine Gene chip in comparisons with the PCR analysis.

Key words: Porcine detection, Porcine Gene Chip™, PCR analysis, gelatin-based products

INTRODUCTION

Bakery industries are one of the important and rapid growth of Halal food industries in Malaysia (Norrakiah *et al*., 2015). Muffin is an individual cup-shaped quick bread made with wheat flour, cornmeal, or the like, and baked in a pan (muffin pan) containing a series of cuplike forms. Possible gelatin being added in muffin is to improve the texture of the muffins. Gelatin is widely used as raw materials and commonly used to improve the quality of a wide variety of foods, beverages, cosmetics and medicines because of its thickening, stabilizing and gelling properties. These leading to the widely use of gelatin in dairy and bakery products especially in cakes, yogurt, cheese and ice creams (Karim & Bhat, 2008; Sahilah *et al*., 2015). Detection of porcine DNA in muffins will help Muslim and Jews community, vegetarians and allergens toward hidden porcine ingredients in processed foods (Tanabe *et al*., 2007) assured about the status of the bakery product by the technology available.

DNA analysis is the technique of choice due to DNA stability which offered polymerase chain reaction (PCR) techniques using DNA amplification of specific target gene.

Mitochondria DNA (mtDNA) is the most common target gene of interest because its DNA is stable and resistant under conditions associated with high temperature, pressure and chemical treatments used in food processing in which DNA has mostly been degraded (Azhana *et al*., 2014; Sahilah *et al.,* 2015). The variations target sequence in mtDNA for porcine DNA detection in food materials are including *cytochrome b* gene, tRNA-ATP8, D-loop, 12S rRNA, 16s DNA, ATP8 and ATP6 (Tartaglia *et al*., 1998; Partis *et al*., 2000; Cheng *et al.,* 2003; Corona *et al.,* 2007; Yoshida *et al*., 2009). The combination of PCR and southern hybridization technique was reported by Sahilah *et al.* (2012) in Halal market surveillance of gelatin capsules in the pharmaceuticals market in Malaysia which targeted mtDNA of *cyt* b gene. The PCR-southern

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hybridization on chip was developed to detect the presence of porcine DNA by hybridizing the denatured biotinylated amplicons with specific probes immobilized onto membrane. The biotin-labeled amplicons bind to streptavidin-alkaline phosphatase and subsequently detected by the colourimetric substrate of nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl-phosphate (NBT/BCIP). The colored signal was captured by Scanner System which allows the species-specific identification at low level of DNA concentration (Sahilah *et al.,* 2012; Aravindran *et al.,* 2014; Sahilah *et al*., 2015).

The concern of prohibited food ingredients being added into foods and beverages has increased consumers awareness thus, sensitive method for detecting the prohibited ingredients in food products is necessary to substantiate any claims regarding the presence or absence of the forbidden ingredients. In this study, the PCR-southern hybridization on chip and PCR analysis was used to detect the porcine DNA in muffins using two different cooking methods of steamed and baked. We compared the sensitivity of both methods in detecting porcine DNA by adding porcine gelatin in muffins.

MATERIALS AND METHODS

Samples for analysis and muffin Preparation

A total of seven gelatin-based samples were purchased from local markets from September to November 2014 and stored at 4°C prior to analysis. The seven samples were three porcine gelatin capsules (C1-C3), three samples of canned porcine meat (BB1-BB3) and a pure porcine skin gelatin sample (GP)(Oxoid, UK) as a set of control. All samples were tested in duplicate.

Fifteen muffin samples were prepared according to the recipe from Chemah *et al*. (2011). The muffin samples were prepared and formulated using six different amounts of porcine gelatin (1, 0.5, 0.1, 0.05, 0.01 and 0% (w/w)). The muffins were then cooked using two different cooking methods which were steamed at 100°C and baked at 170°C for 20 minutes, respectively. Six types of its batter (1, 0.5, 0.1, 0.05, 0.01 and 0% (w/w)) without any heat treatment also were analyzed. All muffin samples were stored at -20°C prior to DNA extraction.

DNA extraction

DNA was extracted by using DNeasy Blood and Tissue Kit (Qiagen, Germany) as described by the manufacturer's instruction. DNA was quantified using MaestroNano® Spectrophotometer (Maestrogen, USA) and the DNA was stored at -20°C until further analysis. All muffins DNA were extracted in triplicate from each source.

Oligonucleotide primers and PCR Amplification

The oligonucleotide primers targeting mitochondria DNA (mtDNA) regions as described by Frezza *et al*. (2008). The sequences of those primers were CYT b-F 5'- ATG ACC AAC ATC CGA AAA TCA C -3', CYT b-R5'- TGC CTA AGA GGG AAC CGA AG -3'. The mtDNA primers were synthesized and supplied by First Base Laboratories (Selangor, MY). Amplification of DNA using primers CYT b-F and CYT b-R targeting the cytochrome b gene at 114 bp as described by Frezza *et al*. (2008).

PCR amplification for southern-hybridization on chip

PCR amplification for southern hybridization was conducted as manufacturer's instruction by using biotin-labeled oligonucleotide primers obtained from Olipro porcine gene chip kit (Olipro™, Malaysia). DNA amplification was performed as described by Sahilah *et al*. (2015).

Southern-hybridization analysis on chip and interpretation of results

The biotinylated amplicons were denatured at 95°C for 10 min and placed into ice block immediately. The hybridization was carried out using PORCINE Gene Chip (Olipro^{TM}, MY) protocols and the detailed was reported by Sahilah *et al*. (2015). Positive detection of porcine DNA is showed by the grey color at two spots in the middle. No color will be formed in the middle of the chip if the result is negative (Sahilah *et al*., 2015).

Detection limit of oligonucleotide primers

The detection limit of all oligonucleotides was examined using Pig Genomic DNA (Novagen®, Germany). The PCR assay condition was similar as described in the PCR amplification as described by Sahilah *et al*. (2015). While, in PCR-southern hybridization analysis, the condition used was similar as described in PCR amplification for Southern-hybridization on DNA Chip.

RESULTS AND DISCUSSION

In the present study, porcine gelatin was added in muffin. The detection of porcine DNA was examined using PCR-southern hybridization on chip and PCR analysis in addressing the lifestyle of choice by Muslim consumers.

Five different concentrations of porcine gelatin 1, 0.5, 0.1, 0.05, 0.01 and 0% (w/w) were treated using two different cooking methods which were steamed at 100°C and baked at 170°C for 20 minutes, respectively. Fifteen muffin samples including its batter (n=5), baked (n=5) and steamed (n=5) muffins were analyzed. There were three

Table 1. Detection of porcine DNA in muffin samples using Porcine Gene Chip and Polymerase chain reaction analysis

Reference code	Muffin cooked samples	Samples positive toward porcine DNA	
		PCR-chip DNA (Cytochrome *b*)	mtDNA of Cytochrome *b* (114 bp)
P1	1% gelatin	+	+
P2	0.5% gelatin	+	+
P3	0.1% gelatin	+	+
P4	0.05% gelatin	+	+
P5	0.01% gelatin	+	–
C	0% gelatin	–	–
P1S	1% gelatin (steamed)	+	+
P2S	0.5% gelatin (steamed)	+	+
P3S	0.1% gelatin (steamed)	+	–
P4S	0.05% gelatin (steamed)	+	–
P5S	0.01% gelatin (steamed)	–	–
CS	0% gelatin (steamed)	–	–
P1B	1% gelatin (baked)	+	+
P2B	0.5% gelatin (baked)	+	+
P3B	0.1% gelatin (baked)	–	–
P4B	0.05% gelatin (baked)	–	–
P5B	0.01% gelatin (baked)	–	–
CB	0% gelatin (baked)	–	–
PC	DNA babi	Valid	Valid
NC	NFW	Valid	Valid
	Total of samples	11/15	8/15
	(%)	73%	53%

*Total samples were 15 excluded C, CB and CS samples. –ve: no gelatin added in the muffin and -ve results for porcine DNA. Valid showed positive and negative result for neither PC nor NC, respectively. + indicated positive results for porcine DNA detection. – indicated negative results for porcine DNA detection.

control muffin samples prepared and tested for porcine DNA namely as C (control for batter muffin), CB (control for baked muffin) and CS (control for steamed muffins). As indicated in Table 1, the highest porcine DNA detected from muffin samples were shown by PCR-southern hybridization analysis on chip. Overall result, PCR-southern hybridization analysis was able to detect 73% (11/15) of muffin samples, followed by PCR analysis targeted *cyt* b gene (53%, 8/15).

The ability of two techniques was compared for porcine DNA detection to batter muffin samples which were not treated with heat. In muffin batters added porcine gelatin, PCR-southern hybridization technique analysis was able to detect all porcine DNA from 1.0, 0.5, 0.1, 0.05 to 0.01% concentrations as indicated in Table 1 and Figure 1. Whereas, the results of PCR analysis for batter muffins using PCR analysis targeted *cyt* b gene showed negative at 0.01% concentrations (Table 1 and Figure 2). Result showed PCR analysis was less sensitive compared to PCR-Porcine gene chip. The *cyt* b primers were chosen in PCR analysis due to its ability to detect porcine DNA in feedstuffs which the DNA is degraded due to the high temperature and hyperbaric treatment applied (133°C/3 bar for 20 min)(Freeza *et al.,* 2008). With high ability to detect porcine DNA in highly treated heat process, these primers were assumed to have high sensitivity to detect the porcine DNA in non-heat treatment. However, the result was otherwise. This may probably due to the sensitivity of both methods to detect the DNA were different. The detection limit results support our findings (Table 2). The detection limit of the PCR-Porcine gene chip primer (Olipro™,

Table 2. Detection limit of Porcine Gene Chip and Polymerase chain reaction analysis (PCR) analysis of SINE primers

Porcine DNA concentration (ng)	PCR-chip DNA (*Cyt* b)	mtDNA of *cyt* b (114 bp)
100	+	+
50	+	+
10	+	+
5	+	+
1	+	+
0.5	+	+
0.1	+	+
0.05	+	+
0.01	+	–
0.005	+	–
0.001	+	–
0.0005	–	–
0.0001	–	–
0	–	–

+ indicated positive results for porcine DNA detection. – indicated negative results for porcine DNA detection.

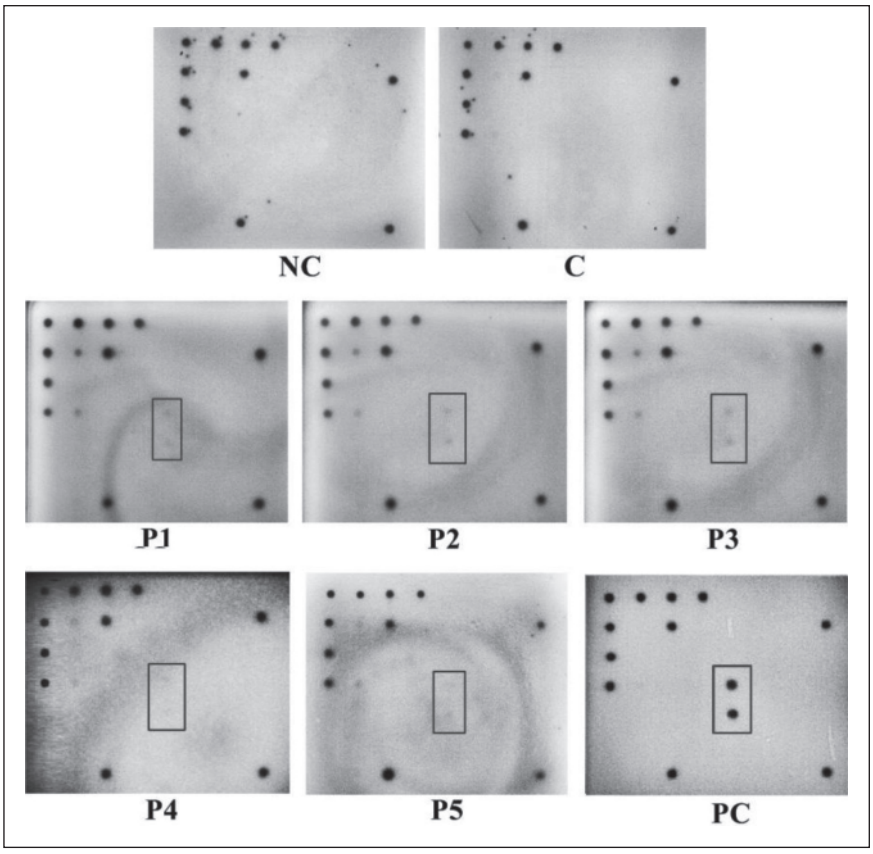

Fig. 1. Gene chip images for the detection of porcine DNA using PCR-southern hybridization analysis in batter muffin samples. NC, negative control; C, muffin with 0% of pork gelatine; P1-P5, muffin added with pork gelatine (1, 0.5, 0.1, 0.05, 0.01 and 0% (w/w)); PC, positive control.

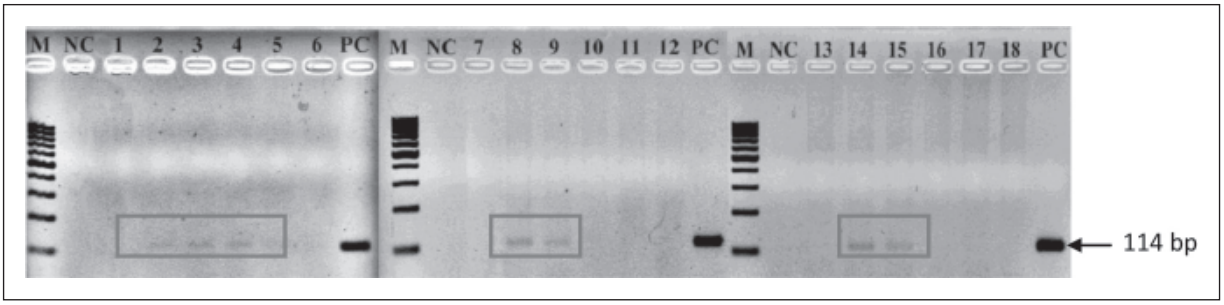

Fig. 2. Amplicons of porcine DNA using PCR assay analysis on 2.5% (w/v) agarose gel. M, 100 bp DNA ladder; NC, negative control; lane 1-6, batter muffins; lane 7-12, steamed muffins; lane 13 -18, baked muffins; and PC, positive control (114 bp).

MY) was 0.001 ng (1 pg) whereas, for primers, PCR analysis was 0.05 ng (*cyt* b). The PCR-Porcine Gene Chip analysis has the advantage to detect at low amplicons concentration by the formation of nitroblue substrate of NBT/BCIP, hybridized with specific probes on chip membrane and give positive result (Sahilah *et al*., 2015). The intensity of two spots in PCR- Porcine gene chip analysis was reducing concomitant with the porcine DNA concentration. Figure 1 shows the interpretation of PCR-Porcine gene chip images for the detection of porcine DNA in batter muffin samples. Porcine DNA positive (PC) showed two spots in the middle of the chip (Figure 1) with eleven spots as the internal control (IC) (Sahilah *et al.,* 2015). There were two other spots which were invisible (negative control) and its position is located above the two positive spots. If the above two spots were positive to porcine DNA, this indicated the chip was contaminated with porcine DNA prior to use. While, less intensity of the bands obtained in PCR-analysis as shown in Figure 2, indicating a low concentration of DNA was extracted.

There were seven known samples examined using both techniques namely capsules (C1-C3), canned porcine meat (BB1-BB3) and porcine gelatin from skin source (GP1). Those samples were referred to as a set of control. Both techniques, either PCR-porcine gene chip or PCR analysis showed a similar level of ability to detect porcine DNA (Figure 3 and 4). This finding was in line with the finding of Sahilah *et al*. (2015) who reported the detection of porcine DNA of capsules and canned porcine meat as controls. While in PCR-analysis the results were expected due to the primers used were claimed able to detect the highly heat samples in hyperbaric condition (Fareeza *et al*., 2005). Therefore, the level of sensitivity may depend on the endurance of DNA in the ingredients or matrix of the samples. These would determine how much damaged the DNA occurred. For example, comparing detection technique between in batter, steamed and baked muffins, using PCR-Porcine gene chip and PCR assay analysis, the sensitivity was affected by temperature treatment. The muffins were the mixture of flour, sugar, egg, margarine and other additives which may assist more damages occurred

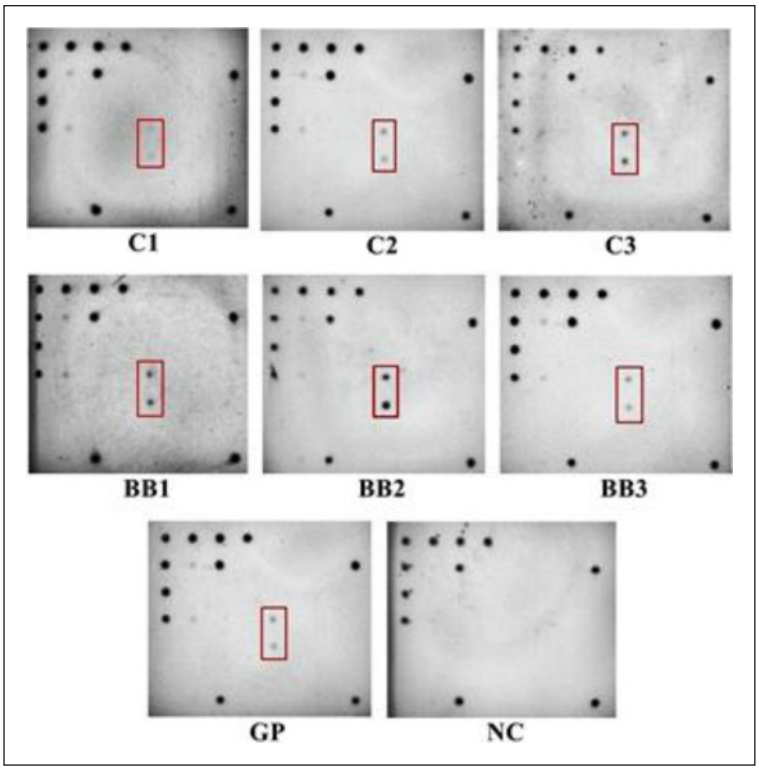

Fig. 3. Gene chip images for the detection of porcine DNA using PCR-southern hybridization on chips for capsule, canned pork meat and porcine gelatin samples. NC, negative control; C1-C3, porcine capsules; BB1-BB3, canned pork meat and; GP, porcine gelatin form skin source.

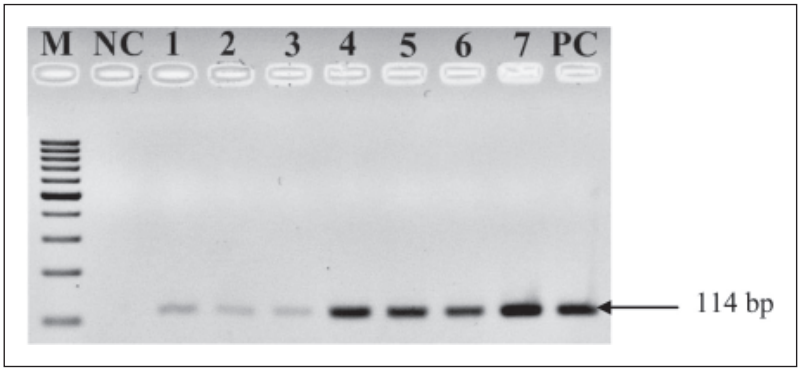

Fig. 4. Agarose gel images of porcine DNA using polymerase chain reaction (PCR) analysis by primers mtDNA. M, 100 bp DNA ladder; NC, negative control; lane 1, porcine gelatin from skin source; lane 2-4, porcine capsule (C1-C3); lane 5 -7, canned meat porcine (BB1-BB3); and PC, positive control (114 bp).

on DNA. These may explain why there were differences in the ability of porcine DNA detection between samples in batter, steamed and baked muffin compared to capsules and canned porcine meat. Gelatin capsules and canned porcine meat were also undergone various steps including heat that might be applied in the manufacturing processes, but these treatments were still not affecting the ability of those techniques to detect the porcine DNA.

CONCLUSION

PCR-Porcine gene chip is sensitive and useful to detect porcine DNA in muffins compared to PCR assay analysis. This approach was able to detect a very low amount of porcine DNA after hybridization analysis with specific probes on chip.

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